



## Sub-lethal photocatalysis bactericidal technology cause longer persistence of antibiotic-resistance mutant and plasmid through the mechanism of reduced fitness cost

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### ABSTRACT

Antibiotic-resistance bacteria (ARB) sustain longevity in environments representing a public health threat recently, but some current bactericidal technologies could eliminate ARB at endpoint of treatment. However, the intermediate process, where bacterial community might be shaped by a complex array of external stressor, was less to be investigated. Herein, the sub-lethal photocatalysis (Sub-PC) bactericidal technology was first applied to validate its effects on the fitness cost and fate of ARB mediated by mutations and antibiotic-resistance gene (ARG) encoded plasmid. Results revealed that the fitness cost of both two types of ARB decreased under Sub-PC conditions as compared with Luria-Broth (LB) broth condition. Plate counting revealed that antibiotic resistance mutant exhibited 6–7 days longer maintenance under the Sub-PC conditions than that under the LB condition. Besides, for plasmid-encoded ARB, the average abundance of ARG in bacteria community was maintained at 0.38 copies for *ctx-1* and 0.58 copies for *mcr-1* under the Sub-PC condition, which were higher than that under the LB condition (0.29 copies for *ctx-1* and 0.45 copies for *mcr-1*). This study suggests that Sub-PC could cause longer-term maintenance of antibiotic resistance and provides an insightful understanding that how anthropogenic sterilization technologies or stressor may alleviate or spread bacterial antibiotic resistance.

### 1. Introduction

The persistence and dissemination of antibiotic-resistance bacteria (ARB) are problematic all over the world and represent a major public health threat recently [1–3]. It is well known that antibiotic resistance of bacteria can evolve via two pathways: chromosomal mutation and acquisition of mobile genetic element [4]. Usually, these processes occurred when certain selection pressures posed by sublethal level of antibiotics were emerging [5,6]. In the presence of antibiotics, the acquisition of antibiotic resistance provides a fitness advantage for ARB in comparison with antibiotic-susceptible competitors. Whereas, in the absence of antibiotics, the antibiotic resistance generally confers disadvantages to bacteria, typically observed as lower growth rate and reduced survival due to the changes in essential genes and/or altered resource usage which termed as fitness cost [7,8]. Based on the mechanism of fitness cost, it is suggested that bacteria should be generally

out-competed by more antibiotic susceptible and higher fitness populations after removing the selective pressure posed by the antibiotics [9,10]. However, after the reduction of clinical usage of some antibiotics, the prevalence of their corresponding resistances among *Escherichia coli* remained in environment [11].

Besides the antibiotics, bacterial communities may also be shaped by a complex array of environmental factors or stressors. It has been reported that, except antibiotics, some other stressors, such as low temperature, resource limitation, could reduce the fitness cost of rifampicin resistance bacterial mutations so that contribute to its persistence in environmental system [12,13]. Besides, different type of growth condition could also confer bacteria different fitness cost. For example, some antibiotic-resistance mutants have shown no fitness cost in laboratory medium but a high fitness cost in mice [14]. Furthermore, some environmental factors could also participate in bacterial adaptive trait, for example, pre-exposure to the stimulus that typically appeared

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early in the ecology could improve the microorganism's fitness when encountered with another stimulus [15]. This might lead to a structure alternation of antibiotic-resistance bacterial community so that influence its antibiotic susceptibilities.

Based on above theory, it is easy to bring us to think that the bactericidal technologies also belong to some kind stressors to bacteria in certain process. For conventional sterilization process, many researches have already revealed that bacteria could be exposed to sub-lethal chlorination and sub-UV stimulation during sterilization process, which could affect structure of bacterial community and even accelerate the evolution or spread of bacterial antibiotic resistance [16,17]. Furthermore, considering the inflexible existence of antibiotic resistance, few studies are focusing on the advanced oxidation technology to eliminate ARB [18–21], among which photocatalytic (PC) technology was pioneered due to its superior oxidative power of generated holes and the reactive oxygen species (ROS) [22–24]. However, most of these researches were carried out under ideal experimental condition with enough treatment time and a single bacterial species so that achieve superior elimination efficiency. However, for the practical application of PC technology, the bacteria might also get away with lethal attack of ROS and only receive sub-lethal oxidation pressure. This process could be defined as Sub-lethal PC (Sub-PC), where bacteria could still grow and in the meanwhile receive sub-lethal attack of ROS, which might make disinfection process counter-productive. Besides, being oxidation burst, both antibiotic-susceptible and -resistance bacteria will part out their aerobically growth ability to withstand oxidative stress and eliminate ROS [25,26], which also has a large impact on their fitness in vitro and in vivo. However, information about the PC stimulation under such circumstances affecting bacterial fitness cost to alleviate or maintain the antibiotic resistance is still limited. Therefore, it is necessary to focus on the intermediate process of PC bacterial inactivation and investigate the alternation of bacterial community structure, when bacteria were undergoing with Sub-PC rather than only consider the bacterial inactivation efficiency at endpoint of treatment.

Based on the above discussion, herein, we mainly investigated how the oxidation pressure posed by Sub-PC affect the persistence of bacterial antibiotic resistance. UV-A (365 nm) and TiO<sub>2</sub> nanotube system was applied to establish Sub-PC stimulation. Two kinds of antibiotic-resistance bacterial strains related to polymyxin (PB) and cefotaxime (CTX) both in type of chromosomal mutation and antibiotic-resistance gene (ARG) plasmid mediated were employed to investigate their fade or maintenance mechanism under Sub-PC stimulation through competitive growth with antibiotic-susceptible strain. The fitness cost of antibiotic-resistance and -susceptible strains were compared through competitive fitness assay and followed by respiration rate measurement, ROS generation and anti-oxidation enzyme activities. Furthermore, during competitive growth, the antibiotic susceptibilities of community were also determined using minimal inhibitory concentration (MIC) method, and the abundance of ARG-encoded plasmid in bacterial community was also quantified through quantify polymerase chain reaction (q-PCR) assay.

## 2. Experimental section

### 2.1. Acquisition of antibiotic-resistance mutants (ARM) and construction of antibiotic-resistance plasmid-encoded bacteria (ARPB)

In this study, two kinds of antibiotics resistance related to CTX and PB were selected, where CTX represents the most widely used  $\beta$ -lactam drug in decades and PB represents the most recently cracked super antibiotics. The detailed information about selected antibiotics is listed in Table S1. Two types of antibiotic-resistance strains including chromosomal mutations and ARG-encoded plasmid were constructed by ourselves. For ARM, parental antibiotic-susceptible strain *E. coli* DH5 $\alpha$  was induced by gradient doubling increased concentration of PB and CTX starting from 1/2 MIC for several times to obtain corresponding

antibiotic-resistance mutants and named as *E. coli* PB-M and *E. coli* CTX-M, respectively. For ARPB, the *ctx-1* and *mcr-1* ARG bearing Inc2 plasmid was introduced to *E. coli* DH5 $\alpha$ , termed as *E. coli* DH5 $\alpha$  (CTX) and *E. coli* DH5 $\alpha$  (PB), respectively. All bacterial strains were collected after incubation in Luria-Bertani (LB) nutrient broth (Sangon, China) at 37 °C for 14–16 h with shaking.

### 2.2. Sub-PC process

A chemostat apparatus was designed to establish the process of Sub-PC bacterial inactivation, 300  $\mu$ L of thin-layer flow through section was linked with a bottle that containing 50 mL of LB medium. The PC section contains TiO<sub>2</sub> nanotube [27] (details show in Fig. S1) loaded onto titanium sheet was used to the PC system under irradiated with 365 nm LED lamp in intensity of 100 mW cm<sup>-2</sup>. A peristaltic pump was employed to drive LB broth circular flow between bottle and PC section (schematic and photo are shown in Fig. S2). In each cycle, the retention time in the PC section and bottle are 1 and 40 s, respectively. The purpose of this design was to ensure that bacteria could receive a certain intensity of PC stimulation, meanwhile to avoid constant oxidation which could lead to extreme bacterial inactivation so that establish the Sub-PC system. Besides, the photocatalyst TiO<sub>2</sub> nanotube used here was to pose a type photocatalytic reaction without specifically materials selection. Control experiments were carried out under identical experimental conditions, except for without PC inactivation process.

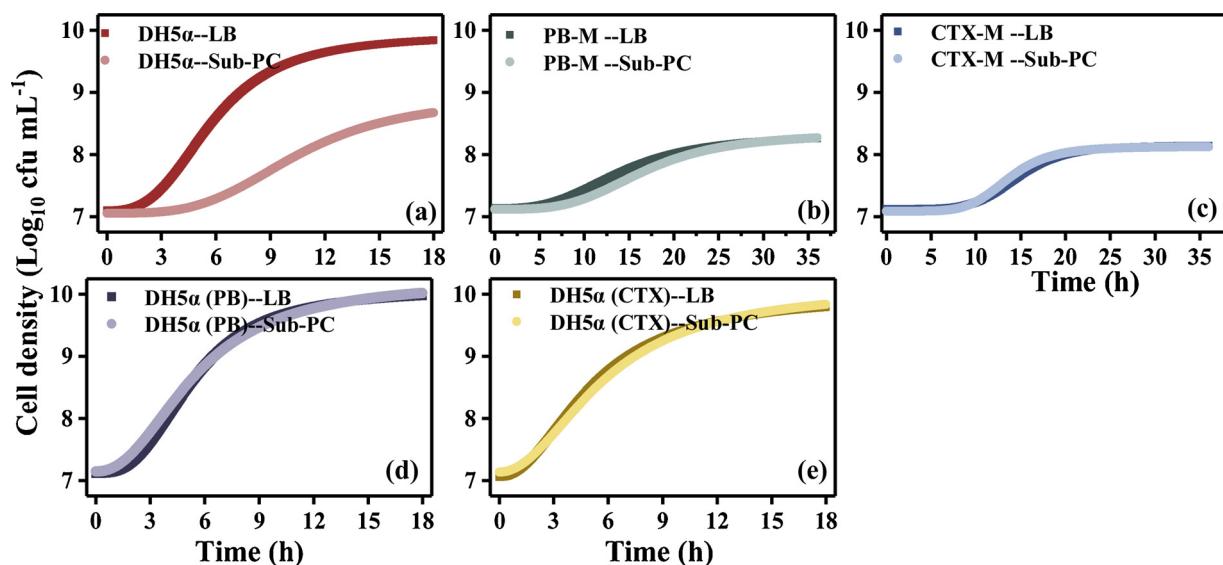
### 2.3. Growth curves and respiration rate assay

To evaluate growth ability of bacteria with and without PC process, the growth curves and respiration rate were assayed. For growth curves, during Sub-PC process, bacteria were sampled at interval of two hours and plate counted to calculate the cell number in triplicate. The bacterial respiration rate was tested using 2,3,5-triphenyltetrazoliumchloride (TTC) and the details are also provided in Supporting information (SI).

### 2.4. Assay of intracellular ROS level and antioxidant enzyme activities

To investigate the bacterial oxidative stress during the growth under the stimulation of Sub-PC, the level of intracellular ROS was determined in different period of cultivation (0, 4, 8, 12, 16 h). The fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was adopted to monitor the intracellular ROS (including H<sub>2</sub>O<sub>2</sub>, 'OH, O<sub>2</sub><sup>-</sup>) levels [28]. The non-fluorescent probe DCFH-DA can freely penetrate into bacterial cells and be hydrolyzed by intracellular esterase and further oxidized by intracellular ROS to form fluorescent 2',7'-dichlorofluorescein, which cannot penetrate into cell membrane. Thus, the probe can be easily packaged in bacterial cells for fluorescent detection. During the assay, a reagent (RSup) increased the concentration of ROS in the cells was used as positive control. The detailed process is provided in our previous work [29].

To reveal bacterial defense to oxidative stress, which represented by the activity changes of intracellular catalase (CAT) and superoxide dismutase (SOD), bacteria were sampled in different growth periods and cell density was adjusted to 10<sup>8</sup> colony-forming unit mL<sup>-1</sup> (CFU mL<sup>-1</sup>). The Bacterial Protein Extraction Kit (Sangon, China) was applied for total protein extraction, and the Modified Bradford Protein Assay Kit (Sangon, China) was applied to measure the concentration of extracted protein before CAT and SOD activity assays. The procedures were followed by protocol of Catalase Assay Kit and Superoxide Dismutase Assay Kit (Beyotime Institute of Biotechnology, China) provided by manufacturer. The detailed procedures were similar to our previous work [30].



**Fig. 1.** Growth curves of antibiotic-susceptible *E. coli* DH5α (a), ARM (PB-M (b), CTX-M (c)) and ARPB ((d) *E. coli* DH5α (PB), and *E. coli* DH5α (CTX)) (e) under LB and Sub-PC system.

## 2.5. Competitive fitness of ARM and ARB

To investigate the influence of Sub-PC on competitive fitness of ARM and ARB, *E. coli* DH5α was mixed with *E. coli* CTX-M/*E. coli* DH5α (CTX) and *E. coli* PB-M/*E. coli* DH5α (PB) by ratio of 1.0 for competitive growth. The competitive fitness of ARM and ARB was determined relative to antibiotic-susceptible *E. coli* DH5α. At baseline and endpoint, the CFU counts on the antibiotics containing plates indicated the number of antibiotic-resistance cells in the mixed cultures. The number of antibiotic-susceptible cells was calculated by subtracting the number of antibiotic-resistance cells from the total cell number revealed by the CFU counts of the plain plates. The competitive fitness W of the antibiotic-resistance strain compared with the antibiotic-susceptible strain was calculated as follows [31]:

$$W = \ln(R^F/R^I)/\ln(S^F/S^I)$$

where R<sup>I</sup> and S<sup>I</sup> refer to the number of antibiotic-resistance and antibiotic-susceptible cells at baseline, respectively; R<sup>F</sup> and S<sup>F</sup> were the number of antibiotic-resistance and antibiotic-susceptible cells at the endpoint. Means of three replicates competition assays were determined.

## 2.6. MIC assay

After each day of bacterial growth under Sub-PC stimulation, the mixture of antibiotic-susceptible bacteria and ARM were harvested at log phase. The MIC was assayed to determine the change of antibiotics susceptibility of mixed community. Briefly, the OD<sub>600</sub> of obtained bacteria were adjusted to 0.1, and then diluted 1:100 into 20 mL of Mueller-Hinton (MH) medium. Approximately 10<sup>6</sup> CFU mL<sup>-1</sup> of bacteria were then moved to 96-well plate, which contains different concentration of antibiotics through doubling dilution for 18 h cultivation at 37 °C. The MIC value was read according to the minimal concentration of well where the bacteria cannot growth.

## 2.7. Abundance of ARG-encoded plasmid in bacterial community through q-PCR assay

To evaluate the persistence of ARG plasmid in bacterial community, the abundance of ARG plasmid in mixed bacterial community was determined. The average copies number of ARG per cell was calculated to represent the abundance of ARG plasmid in bacterial community level.

The q-PCR was applied to quantify the copies number of ARG and single-copy gene *dks* (reference gene), where the copies number of ARG represented the number of ARG-encoded plasmids, and the copies number of *dks* gene represented total number of bacteria include ARB and antibiotic-susceptible strain. The average copies number of ARG per cell was obtained by ratio of copies number of ARG to *dks* gene. Aliquot of 1 mL of bacteria suspension was collected and centrifuged at 8000 rpm to remove LB broth. Then, the sodium laurylsulfonate was added and incubated in 37 °C for 30 min to remove extracellular DNA with centrifuge. After that, bacterial intracellular genome DNA was extracted through Total genome DNA extraction Kit (Sangon, China).

For q-PCR quantification, the standard curves were established firstly. Specifically, following protocol optimization (Table S2 for q-PCR primer information), q-PCR amplicons of *ctx-1* and *mcr-1* ARGs were purified using a SanPrep Column DNA Gel Extraction Kit (Sangon, China). Standard curves were obtained for each q-PCR run using 10-fold serial dilutions (10<sup>0</sup> to 10<sup>8</sup> copies) of the amplicons. After validation and optimization of the protocols of SYBR green q-PCR, the sample and standards were conducted in 96-well plates with a final volume of 25 μL. The detailed reagents, concentrations, and reaction procedures of qPCR and the calculation method of copies number of ARGs are provided in SI. Each reaction was run in triplicate, and the q-PCR efficiency of each gene ranged from 90% to 110%, with R<sup>2</sup> ≥ 0.99 for all standard curves.

## 2.8. Statistical analysis

Analysis of variance (ANOVA) were used to determine significant differences in bacterial competitive fitness, and the P-value no more than 0.05 (P < 0.05). Quantification of ARG abundance under Sub-PC and LB condition, relative respiration rate, relative ROS generation as well as anti-enzyme activities of antibiotic-resistance strains were replicated three times, and the results are expressed as means ± standard deviations.

## 3. Results and discussion

### 3.1. Comparison of growth curves under Sub-PC and LB system

To investigate the effect of Sub-PC stimulation on bacterial growth, the growth curves of two kinds of ARM (*E. coli* PB-M and *E. coli* CTX-M) and ARPB (*E. coli* DH5α (CTX) and *E. coli* DH5α (PB)) as well as

antibiotic-susceptible strain *E. coli* DH5 $\alpha$  were determined firstly. As shown in Fig. 1a, after 1 h of lag phase, the antibiotic-susceptible *E. coli* DH5 $\alpha$  grew faster and reached  $8.7 \times 10^9$  CFU mL $^{-1}$  of cell density after 16 h of inoculation in LB broth. Comparatively, with the addition of Sub-PC stimulation, the bacterial growth rate slowed down extremely with prolonged lag phase and the maximal cell density in stationary phase decreased to  $6.5 \times 10^8$  CFU mL $^{-1}$ , which might be due to the adverse condition posed to bacteria by generated ROS in PC section. Corresponding to our previous work, the high level of ROS could cause oxidative damage to bacterial cells, for example, reducing ATP generation as well as respiratory rate, and even destroying bacterial intracellular components such as protein and DNA so that block the normal growth of bacteria [29,32].

Compared with antibiotic-susceptible *E. coli* DH5 $\alpha$ , it is harder for ARM to grow both in LB and Sub-PC condition. As shown in Fig. 1b and c, the lag phase was extended to approximately 6 h with no any of increase of cell numbers both for *E. coli* CTX-M and *E. coli* PB-M in LB cultivation. Afterwards, the cell densities reached  $2.6 \times 10^8$  CFU mL $^{-1}$  for *E. coli* CTX-M and  $4.7 \times 10^8$  CFU mL $^{-1}$  for *E. coli* PB-M till 36 h of growth. It is reasonable for slow growth of ARM under the LB condition, since ARM usually mutates in essential genes and/or alters resource usage leading to a fitness cost and slow growth when ARM transfer to normal condition where the selection of antibiotics was removed [7]. However, different from the response of antibiotic-susceptible *E. coli* DH5 $\alpha$ , a strange phenomenon was found for ARM under the identical conditions. That is, when Sub-PC stimulation was introduced to bacterial cultivation, there is no observable decline of the growth rate for ARM, which is very similar with that in LB system (Fig. 1b and c). It suggested that the oxidation pressure posed by Sub-PC stimulation did not affect the growth situation of ARM. The reason might be that, mutations occurred in ARM that selected by antibiotics could improve bacterial defense system when the ARM faced to subsequent stimulus through the mechanism such as overexpression of efflux pumps [33] or facilitation bacterial global stress response [34].

Unlike the ARM, which acquired antibiotic resistance through chromosome mutations to alter its metabolic patterns and certain functions, the ARPB could acquire antibiotic resistance by obtaining ARGs directly through horizontal transfer without genetic change. It was supposed that the acquisition of ARG might also confer bacteria a subtle fitness cost, and the growth rate of ARPB cannot catch up with antibiotic-susceptible bacterial strain [35]. However, in an inverse trend, both in LB and Sub-PC systems, *E. coli* DH5 $\alpha$  (CTX) and *E. coli* DH5 $\alpha$  (PB) showed superior growth rates to antibiotic-susceptible *E. coli* DH5 $\alpha$  without differentiable lag phase (Fig. 1d and e), indicating that the existence of ARG might improve bacterial fitness. Besides, our result was corresponding to some other researches, where some ARG-encoded plasmids could confer fitness advantage to bacteria in vitro in the absence of selected antibiotics pressure [36,37].

From the results of the bacterial growth curves, it could be found that the Sub-PC stimulation was able to affect the growth situation of antibiotic-susceptible *E. coli* DH5 $\alpha$  to a large extent with decreased growth rate. In contrast, the growth situation of ARM showed more stability and there is no distinguished difference between in LB and Sub-PC system. Thus, we can infer that the growth competitiveness gap between the ARM and antibiotic-susceptible *E. coli* DH5 $\alpha$  was narrowed in Sub-PC system so that reduce fitness cost of ARM. Besides, with acquisition of ARGs, the ARPB shows a better fitness than antibiotic-susceptible *E. coli* DH5 $\alpha$ , which might promote persistence of ARG-encoded plasmids in bacterial community.

### 3.2. Relative respiration rate of bacteria during Sub-PC system

To further verify the bacterial growth rate, the respiration rate, which represents bacterial energy metabolism, was also assay. As shown in Fig. 2, with the addition of Sub-PC stimulation, the relative respiration rate of antibiotic-susceptible *E. coli* DH5 $\alpha$  decreased

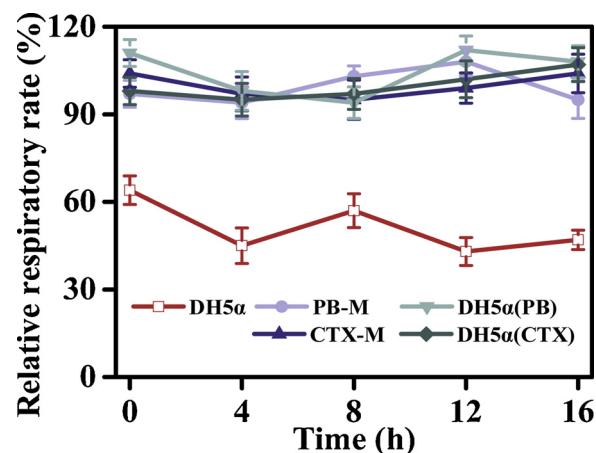


Fig. 2. Relative respiration rate of bacterial growth with Sub-PC stimulation compared with normal condition. Data are presented as the mean  $\pm$  s.d. of three independent experiments.

dramatically to 40% as compared with normal growth condition. The loss of bacterial respiration rate might be due to adverse effects by the generated ROS on cell membrane proteins, which play an important role in respiration chain [38], so that reduce bacterial growth rate. Furthermore, coincided with the results of growth curves, the Sub-PC did not caused distinguished differences in relative respiration rate between ARM and ARPB (Fig. 2), suggesting that the mutations or ARG-encoded plasmid might confer advantages to the bacterial cells, in essence allowing bacteria to perform intrinsic functions more efficiently and stay in more stable state when ARB and ARPB face to subsequently fluctuated growth condition [34,36].

### 3.3. Oxidative stress response during growth processes with stimulation of Sub-PC

Under the stimulation of PC, bacteria could suffer from an oxidation pressure by the generated ROS, which could destroy bacterial membrane and further penetrate into the cell to disturb bacterial metabolism, subsequently leading to increase level of intracellular ROS and bacterial oxidative stress responses [32,39]. To investigate how did the ARM, ARPB and antibiotic-susceptible *E. coli* DH5 $\alpha$  response to the oxidation pressure, the intracellular ROS formation and activities of anti-oxidative enzymes (CAT and SOD) were also assayed during whole bacterial growth period under Sub-PC system. As shown in Fig. S2, relative to normal condition, the intracellular ROS in antibiotic-susceptible *E. coli* DH5 $\alpha$  increased obviously under Sub-PC condition, indicating that the intracellular oxidative stress was aroused under this circumstance. To maintain bacterial intracellular redox equilibrium for normal metabolic activity, the content of both anti-oxidative enzymes, CAT and SOD, increased (Figs. S3 and S4) and served as scavengers of ROS to eliminate oxidative damage in bacterial intracellular. In addition, this elimination process of oxidative stress might consume part of metabolic energy, which could account for the decreased bacterial growth.

In contrast, for the ARM and ARPB, there is no obvious oxidative stress responses occurred under the Sub-PC condition (Fig. S3), and the level of intracellular ROS fluctuated slightly (Fig. S3), indicating that ARM and ARPB are more endurable to the oxidation pressure and keep in redox equilibrium invariably. Correspondingly, the activities of CAT and SOD also remain unchanged during the whole bacterial growth period under Sub-PC stimulation (Figs. S4 and S5). It is corresponding with above mentioned results that Sub-PC cannot affect the growth abilities of ARM and ARPB obviously, which might due to more effectively intrinsic function mediated by bacterial mutations or ARG-encoded plasmid to make bacteria stay in more stable state when ARM

and ARPB face to subsequent fluctuated growth condition [34,36].

Usually, the bacterial fitness cost was depended on the growth gap between ARM/ARPB and susceptible strain, and the greater the growth gap is, the larger fitness cost of ARM/ARPB is exhibited. Nevertheless, with the addition of Sub-PC stimulation, unlike the dramatic growth impairment to *E. coli* DH5 $\alpha$ , no observable growth impairment was caused to ARM/ARPB, which could narrow the bacterial growth gap between *E. coli* DH5 $\alpha$  and ARM/ARPB. Based on this analysis, we can also infer that, compared with normal LB growth condition, the fitness cost of ARM/ARPB might decrease with addition of Sub-PC stimulation.

### 3.4. Relative competitive fitness of ARM and ARPB during competitive growth with antibiotic-susceptible bacterial strain

Based on the above obtained results, we hypothesized that Sub-PC stimulation could narrow down the fitness gap between *E. coli* DH5 $\alpha$  and ARM/ARPB. In another word, the fitness cost of ARM/ARPB could be reduced by addition of Sub-PC stimulation and persisted longer time during their competitive growth with antibiotic-susceptible *E. coli* DH5 $\alpha$ . To validate this hypothesis, the competitive growth (with/without Sub-PC stimulation) was employed to investigate the relative competitive fitness of ARM /ARPB to antibiotic-susceptible *E. coli* DH5 $\alpha$ . As shown in Fig. 3a, the value of relative competitive fitness with Sub-PC stimulation increased dramatically from 0.33 and 0.28 (without Sub-PC as control) to 0.64 and 0.71 for *E. coli* CTX-M and *E. coli* PB-M ( $p < 0.05$ ), respectively, indicating that growth competitiveness of ARM was improved from 33% to 64% (*E. coli* CTX-M) and 28% to 71% (*E. coli* PB-M) relative to susceptible *E. coli* DH5 $\alpha$ . This result coincided with our hypothesis that the competitiveness fitness of ARM was improved with the Sub-PC stimulation due to the narrowed growth gap between ARM and antibiotic-susceptible *E. coli* DH5 $\alpha$ .

Furthermore, the competitive growth between ARPB and antibiotic-susceptible *E. coli* DH5 $\alpha$  was also carried out. As shown in Fig. 3b, both

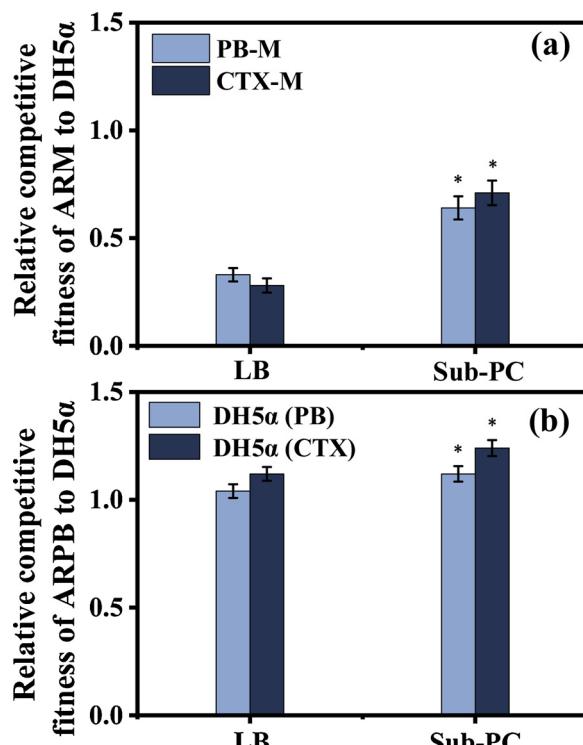


Fig. 3. Relative competitive fitness of ARM (a) and ARPB (b) to antibiotic-susceptible *E. coli* DH5 $\alpha$  during the growth with and without Sub-PC stimulation. Significant differences were tested with one-way ANOVA analysis, \* $P < 0.05$ .

in LB and Sub-PC system, the values of relative competitive fitness were both exceed 1.0, indicating that the growth competitiveness of ARB (*E. coli* DH5 $\alpha$  (CTX) and *E. coli* DH5 $\alpha$  (PB)) was superior to antibiotic-susceptible *E. coli* DH5 $\alpha$ . This might be explained that the ARG-encoded plasmid could improve bacterial growth ability and show enhancement of adaptation. Furthermore, there is an observable difference in competitive fitness between LB and Sub-PC system, where the competitiveness of ARPB was slightly improved with the addition of Sub-PC stimulation, indicating that the ARG-encode plasmid used in this study could not only improve bacterial competitive fitness but also enhance bacterial competitiveness when ARPB face to subsequent Sub-PC stimulus.

It has been reported that, when ARM and ARPB disseminate to bacterial community in environment in absence of antibiotics, based on the mechanism of fitness cost, which the competitive fitness of ARM and ARPB are weaker than antibiotic susceptible bacterial strain, the ARM and ARPB could be out-competed by antibiotic-susceptible bacterial strains, leading to reverse of antibiotic resistance due to declined proportion of antibiotic-resistance strains [7]. However, according to our study, with addition of oxidation pressure posed by Sub-PC stimulation, the increased competitive fitness might slow down the reverse of antibiotic resistance in bacterial community. Therefore, it brings us to consider that the antibiotic resistance of bacteria might be maintained for a long term by Sub-PC stimulation even without existence of antibiotics in the environment.

### 3.5. Long-term maintenance of antibiotic-resistance mutant and ARG during competitive growth under Sub-PC system

To investigate whether the Sub-PC stimulation could contribute to long-term maintenance of antibiotic resistance of bacteria or not, individual ARM or ARPB was competitively co-cultured with antibiotic-susceptible *E. coli* DH5 $\alpha$  continuously for several days to study the variation of antibiotic sensitive and abundance of ARG. As shown in Fig. 4, under the normal culture condition without Sub-PC stimulation, the MIC of bacterial community, containing *E. coli* CTX-M and *E. coli* PB-M, to its corresponding antibiotics CTX and PB declined rapidly from 256 to 0.1 and 1.0 ppm, respectively. However, with addition of Sub-PC stimulation, the MIC of bacterial community was maintained steadily in early days of competitive growth and declined more slowly than that under LB condition, indicating that the proportion of ARM was persist and kept in high abundance in bacterial community. Furthermore, the existence of antibiotic-resistance bacterial strains was also counted on antibiotics containing plate. As shown in Fig. 5, after four and six days of competitive growth in LB broth, the existence of *E. coli* CTX-M and *E. coli* PB-M were faded away with no observation of bacterial colony. Comparatively, with the addition of Sub-PC stimulation, the bacterial colony of both *E. coli* CTX-M and *E. coli* PB-M persist till 9 and 12 days, respectively, of competitive growth, indicating that the ARM show an improved competitiveness in Sub-PC system than that in LB. Some researchers also reported that long-term induction of ROS could cause the increase of bacterial mutation and generate antibiotic-resistance bacterial strains [5,33]. Therefore, the antibiotic-susceptible *E. coli* DH5 $\alpha$  might be also induced to mutate into ARM by Sub-PC stimulation in this study. Nevertheless, after seven days of Sub-PC induction, the sensitive of *E. coli* DH5 $\alpha$  to antibiotics CTX and PB were unchanged with no variation of diameter of inhibition zone (Fig. S5), indicating that Sub-PC stimulation cannot contribute to antibiotic resistance of bacteria directly within a matter of several days. Therefore, the mechanism of long-termed maintenance of antibiotic resistance by bacteria in this study was due to the narrowed down the growth competitiveness gap between ARM and antibiotic-susceptible *E. coli* DH5 $\alpha$ , subsequently contributed to longer antibiotic resistance persistence of ARM. Similar to this study, long-term exposure to chloride disinfectant could also change the structure of microbial community and increase bacterial antibiotic resistance [40]. Considering this maintenance of

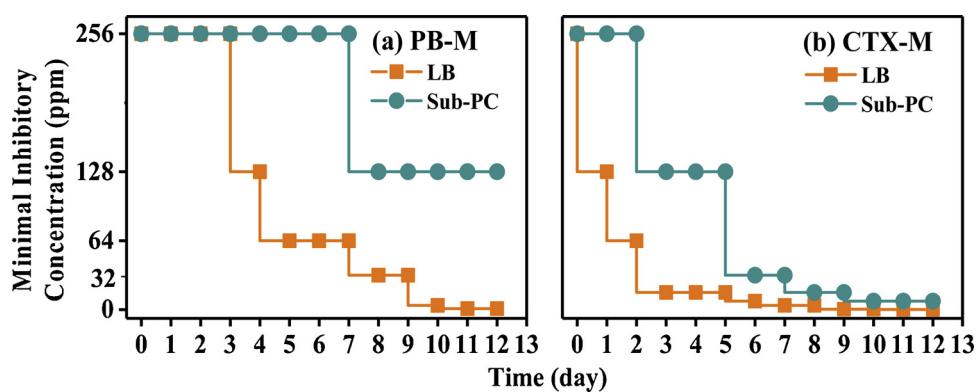


Fig. 4. Reverse of MIC of bacterial community that containing CTX-M (a) and PB-M (b) during competitive growth with and with the addition of Sub-PC stimulation.

bacterial antibiotic resistance, it could explain why the antibiotic resistance of bacteria is hard to be eliminated in environmental system. Furthermore, except the oxidation pressure, plenty of other threatening conditions might also relieve the competition between antibiotic-resistance and antibiotic-susceptible bacterial strains, so that pose reverse obstacle of antibiotic resistance.

Furthermore, the persistence of ARG in bacterial community which represented by average copies number of ARG per cell were also quantified by q-PCR. Based on the abovementioned results, which the acquisition of ARG-encoded plasmid could confer fitness advantage to the bacteria, it is supposed that the abundance of ARG-encoded plasmid could increase profoundly in bacterial community. However, our obtained results showed opposite trend (Fig. 6a and 6b), where the average copies number of ARG per cell decreased quickly in early period of competitive growth in LB system from 0.65 to 0.52 and 0.54 to 0.35 for *E. coli* DH5 $\alpha$  (PB) and *E. coli* DH5 $\alpha$  (CTX), respectively. After

that, the average copies number of ARG per cell was plateaued without obvious decrease or increase (0.29 copies for *ctx-1* and 0.45 copies for *mcr-1*). It might be explained that there exist two forces to mutually restrict, including that 1) ARG-encoded plasmid confers advantage to bacterial competitive fitness to a certain extent; 2) overmuch copies number of ARG-encoded plasmid in bacterial intracellular consumes much energy and confers fitness cost to the bacteria so that urge bacteria to discard parts of ARG plasmid through the segregation instability or some other process [41]. Therefore, after several days of competitive growth of mixed ARPB and susceptible strain *E. coli* DH5 $\alpha$ , the average copies number of ARG per cell was reach a balance level. Furthermore, to compare the differences between LB and Sub-PC condition. It is obvious that the Sub-PC stimulation slowed down the decrease of average copies number of ARG per cell and the ARG-encoded plasmid sustained in higher level in bacterial community (0.38 copies for *ctx-1* and 0.58 copies for *mcr-1*) (Fig. 6a and b). Similarly, it has been

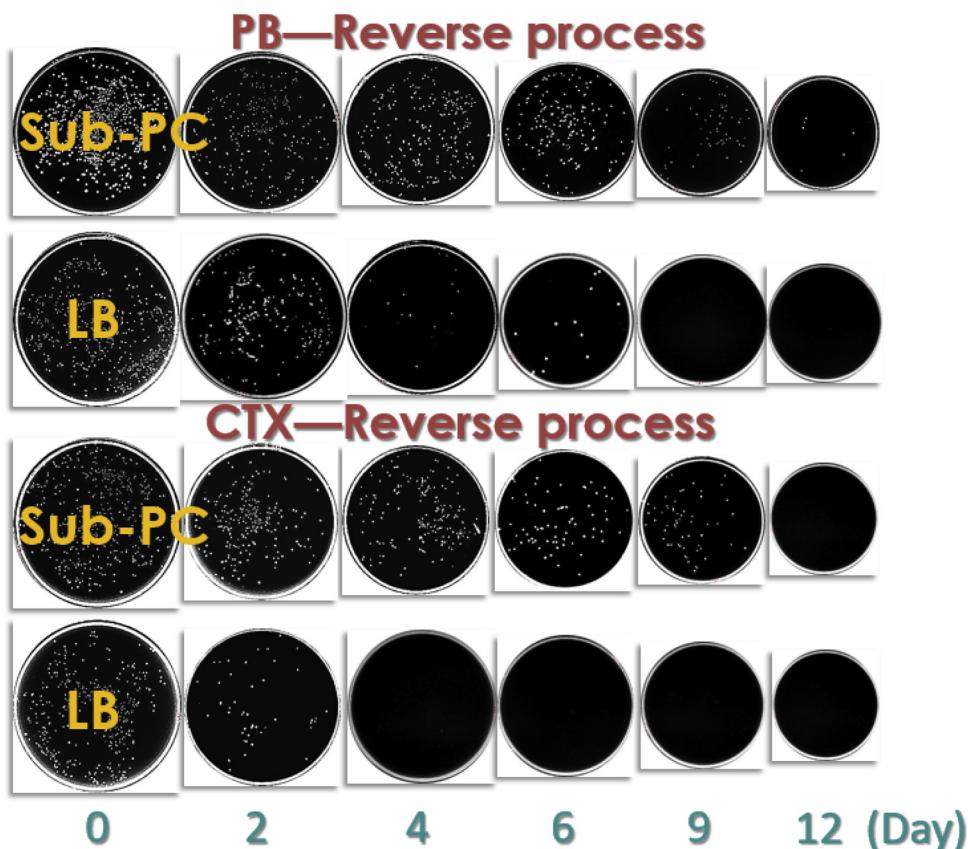
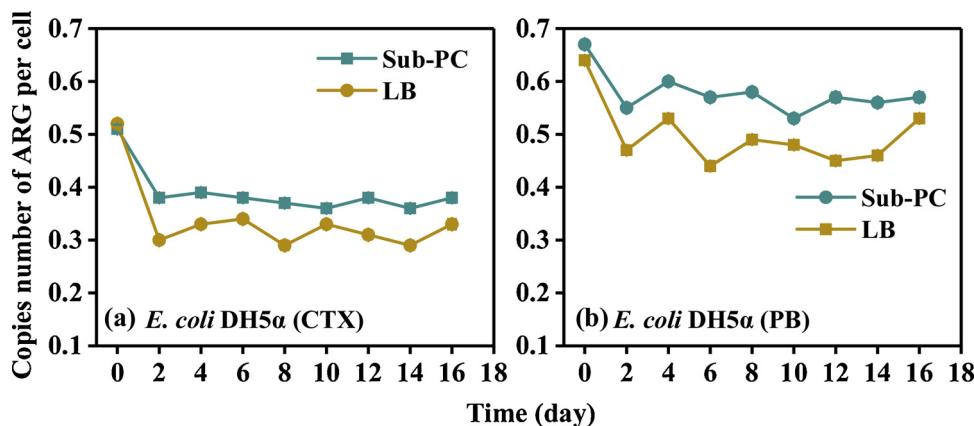


Fig. 5. The existence of ARM counted on antibiotics-containing plate during continuous competitive growth under normal condition and Sub-PC stimulation.



**Fig. 6.** Abundance of ARG-encoded plasmid (*MCR-1(a)*, *CTX-1(b)*) during competitive growth with antibiotic-susceptible *E. coli* DH5 $\alpha$  through q-PCR quantification. Data are presented as the mean  $\pm$  s.d. of three independent experiments.

reported that besides antibiotics, some other environmental factors such as heavy metals or biocides could also worsen the crisis of antibiotic-resistance by posing co-selection of ARB [42–45]. The mechanism of this selection is based on similar function of antibiotics to screen more antibiotic-resistance bacterial cells. However, in this study, the driving force to ARG accumulation was based on the enhancement function of ARG-encoded plasmid, which might reinforce bacterial intrinsic functions to improve bacterial growth competitiveness.

#### 4. Conclusions

In conclusion, this study provides an evidence that the oxidation pressure posed by Sub-PC could maintain the bacterial antibiotic resistance for a long term through reducing the fitness cost of antibiotic-resistance bacterial strains. With the addition of Sub-PC stimulation, the ARM shows more durable to the oxidation pressure, and maintains its normal respiration rate as well as redox equilibrium as compared with susceptible *E. coli* DH5 $\alpha$ . These could close the growth gap between ARM and antibiotic-susceptible bacterial strain in Sub-PC system as compared with LB condition, so that retard the process of out-competed by antibiotic-susceptible bacterial strain, finally lead to long-term maintenance of bacterial antibiotic resistance. Besides, with Sub-PC stimulation, the abundance of ARG in bacterial community maintained in higher level and persist longer time than that under the LB condition, which might strengthen the problem of antibiotic resistance spread. This study revealed the potential side-effect of PC in bacterial inactivation and highlighted the necessity to focus on intermediate process investigation when designing appropriate strategies to eliminate antibiotic resistance problem.

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#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.apcatb.2019.01.041>.

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